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Effect of caffeine supplementation during treadmill exercise on hippocampal genes expression levels in adolescent rats

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ABSTRACT

Objectives: The usage of caffeine, a psychostimulant that is included in many foods and drinks, is rising, especially among young people. Also, caffeine works as an ergogenic substance during exercise to improve performance, and strength. In this study, our aim was to investigate the effect of caffeine consumption and exercise on hippocampal learning and memory functions in early life.

Methods: Postnatal 28 days old Wistar albino male rats (n = 28) were randomly divided into 4 groups; control group (C), caffeine group (Cf), exercise group (E), caffeine+exercise group (CfE). Caffeine was dissolved in drinking water (0.3 g/L) and the treadmill exercise was applied 3 days a week. Following, the rats were applied to Morris Water Maze Test (MWMT) and open field test. N-methyl-D-aspartate (NMDA) receptors NR2A, NR2B, and brain-derived neurotrophic factor (BDNF) gene expression levels were investigated in hippocampus tissue by RT-PCR.

Results: In MWMT, there was no significant difference in terms of learning and memory functions and hippocampal gene expression levels of the groups ($p > 0.05$). In the open field test, the time spent in the center was decreased in the CfE group, and the number of entries to the center was decreased in the E and CfE groups compared to the control group ($p < 0.05$).

Conclusions: We assumed that caffeine given with exercise application caused anxiety behavior but did not affect learning and memory. There is a need for new studies investigating the effect of caffeine on exercise with different doses and durations depending on age.

Keywords: Caffeine, exercise, hippocampus, learning, memory

Caffeine is a psychostimulant substance that is frequently consumed all over the world and increases alertness by showing a stimulant effect on the peripheral and central nervous systems [1]. Caffeine affects central nervous system stimulation, increases metabolism and respiratory rate, induces diuresis, and increases blood pressure by antagonizing adenosine receptors. Thus, it prevents drowsiness and increases alertness [2]. Caffeine performs its psychostimulant effect through the adenosine A1 receptor. Adenosine A1 receptors are abundant in some brain regions, such as the hippocampus, cortex, cerebellum, and hypothalamus [3]. It is known that caffeine has a neuroprotective effect in various neurodegenerative diseases and

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is protective against neurotoxicity in Alzheimer's. Moreover, adenosine and N-methyl-D-aspartic acid (NMDA) receptors may be responsible for this neuroprotective mechanism [4]. For instance, Oliveriora and colleagues showed that subchronic caffeine treatment can reduce the hyperlocomotion and cognitive deficits caused by NMDA receptor antagonists in mice [5]. Together with caffeine, physical activity has positive effects on cognition and brain function at various levels.

Exercise can improve cognitive functioning (such as executive function in old age or mental ability in children with educational disabilities), reduce stress levels, and ward off feelings of anxiety and depression [6]. Exercise is individually planned and known as a structured physical activity that is done voluntarily to be fit and healthy [7]. Exercise protects neurons from various brain injuries, activates neuronal cells, promotes neurogenesis, increases brain plasticity, and improves cognitive function [8]. Exercise increases NMDA receptor expression in the hippocampus [9, 10]. In a study with Alzheimer's transgenic mice, they found that the NMDA receptor subunit NR2B levels were decreased. They also reported that NR2B levels increased significantly after exercise administration, thereby attenuating NMDA receptor damage and improving cognitive memory [11]. Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophic factor and stimulates the brain with exercise [12]. It has been reported that BDNF prolongs the lifespan of neurons, preserves their integrity, stimulates neurogenesis, strengthens learning, and protects cognitive functions during aging [13]. Exercise affects BDNF expression through both direct and indirect mechanisms [14].

Caffeine is an ergogenic supplement that increases cognitive function and ameliorates the performance of exercise by increasing endurance and strength [15, 16]. It has been reported that caffeine consumption and physical exercise that is applied during the adolescent development period have the potential for improving behavioral disorders and stimulating neuroplasticity in attention deficit hyperactivity disorder (ADHD) [17]. Human studies investigate the behavioral effects of caffeine in adults in particular [18]. However, there are fewer studies in the literature evaluating the safety and effects of caffeine in children and adolescents compared to adults. Based on this information, the purpose of this study was to look into the effects of caffeine and/or physical exercise on learning and memory in adolescent rats using behavioral tests and hippocampal NR2A, NR2B, and BDNF gene expression levels.

METHODS

Experimental Protocol

All experimental procedures were carried out according to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. The ethical approval of the study was taken from the Experimental Animal Ethics Committee of Mersin University (Approval No: 2021/21). Wistar albino male rats (n = 28, 4 weeks old postnatal) were randomly assigned into four groups: control group (C), caffeine group (Cf), exercise group (E), caffeine + exercise group (CfE). The rats were fed with tap water and Purina rodent chow ad-lib. All applications and tests were carried out in the physiology laboratory of the Mersin University Medicine Faculty. During the experiment, the temperature of the room where the animals were kept was adjusted to 23 ± 2ºC. The rats' consumed the amount of water measured daily, and their weight and the amount of feed were followed up weekly.

Caffeine Treatment

Caffeine is an anhydrous form (Sigma Aldrich, Saint Louis, MO, USA, lot: r068K8730V) was administered via drinking water (0.3 g⁄L) chronically for 4 weeks and prepared daily [19, 20]. The daily consumption of caffeine by each rat was ensured to be nearly 16-20 mg. The amount of water consumed by rats in the caffeinated groups for 4 weeks was found similar to the control group.

Treadmill Exercise Application

Exercise application was started from the 28th day of postnatal period (PND). The exercise protocol was performed on a treadmill apparatus with 2 sections, 3 times a week for 4 weeks. Exercise intensity is ~ 0.5 km/h for 4 weeks; slope increase was not applied [21]. Before starting the exercise application, the rats were kept on the treadmill for 10-15 minutes to familiarize themselves with the environment, and then the exer-

cise application was started. Exercise application was performed as 20 min/day in the first week, 50 min/day in the second week, 60 min/day in the 3rd week, and 60 min/day in the 4th week. The learning memory performances of the rats whose exercise application was completed were measured with the Morris Water Maze Test (MWMT), and their anxiety levels were measured with the open field test.

Testing Spatial Learning and Memory Using the Morris Water Maze Test (MWMT)

A circular stainless-steel tank that has a diameter of 1.5 m and 0.6 m in depth was used in the MWMT. The tank was filled with water to a depth of 0.5 m. The water and room temperature were adjusted to the temperature of 22 ± 1°C. A curtain ornamented with several marked visual cues surrounded the tank. Inside the tank, there was a circular platform that has a diameter of 15 cm. To record the swimming track of the test animals, a camera was used placed above the tank. The behavioral data were recorded and analyzed by a visual analysis system (EthoVision, Noldus Information Technology, Wageningen, NL). The parameters recorded during the experiments included latency, the path length of rats to reach the platform in meters (m), swimming velocity in cm/s, and time spent in the targeted quadrant. All experiments were conducted between 9:00 a.m. to 13:00 for 6 days [20].

MWMT was performed with 4 days of learning, with the first day of habituation, and a memory test on the last day. The visible platform (1.5 cm above the water surface) on the first day of the experiment, the hidden platform at 2-5 days (1.5 cm below the water surface), and the platform was not used on the 6th day. On the habituation day, the rats were released to the tank, facing the wall. The rat was expected to find the platform within 60 seconds. The rats that could not find the platform were guided by the researcher with the help of a plastic stick, and they were allowed to find the platform and stay on the platform for 5 seconds. In the learning days (2-5). The platform was fixed to the southeast quadrant. The rats were released into the water 4 times a day from 5 different locations and were expected to find the platform in 60 seconds. During the learning days, the time to find the platform (latency, sec), total distance traveled (cm) and swimming speed (cm/sec) were recorded and analyzed. On the 6th day, the last day of the experiment, the platform was removed from the water and the rats were released into the water with a single shot in the north direction, and recording was made for 60 s. At the end of this period, the ratio of the average time spent by the rats in the southeast quadrant, where the platform was previously located, to the total time was calculated.

Testing Anxiety Level Using the Open-Field Test

The open field test is used to measure locomotor activity and anxiety behavior of experimental animals entering a new environment [22]. The open-field apparatus (100 × 100 × 40 cm) was a black opaque plexiglass open area that was divided into the central zone and the peripheral zone using the software. On the test day, rats were placed in the center to explore the environment for 10 min. At the end of the test, the animal was returned to its home cage and the maze was thoroughly cleaned with 20 % ethanol. The total distance moved and time spent in the center was measured with the Noldus Ethovision tracking system. The room temperature was maintained at 21 ± 2 ◦C, and behavioral tests were performed at this temperature [23].

Determination of Hippocampal NR2A, NR2B, and BDNF Gene Expression Level

The rats in all groups were decapitated under ketamine xylazine anesthesia and their brain tissues were removed. The brain was placed in PBS (Phosphate Buffered Saline) solution and hippocampus tissue was isolated. Isolated right and left hippocampus tissues were stored at -80°C to determine gene expression levels by RT-PCR. Expression levels of NR2A, NR2B, and BDNF genes in the hippocampus were investigated.

Total RNA was isolated from the hippocampus tissues with RNeasy Lipid Tissue Kit (Qiagen Inc., Valencia, CA) / TRIZOL (Invitrogen). Isolated RNA was dissolved in an RNase-free solution for cDNA synthesis. 4 µl total RNA extraction was used as a template for the synthesis of cDNA. cDNA was obtained by using a High-Capacity cDNA Reverse Transcription Kit (LifeTech Cat. no. 4368814) Individual reactions were carried out using thermal conditions (Bioer, Gene Pro Thermal Cycler): 25 ◦C for 10 min, 37 ◦C for 120 min, 85 ◦C for 5 s, and 4 ◦C for 1 min. RT- PCR was run on an Applied Biosystems Viia 7 using Taqman GE Master Mix. β-actin was used as a housekeeping gene that is generally preferred in neurological re-

search. The reaction was carried out using 40 amplification cycles of 50 ◦C for 2 min, 95 ◦C for 10 min, 95 ◦C for 15 s, 60 ◦C, for 1 min, and 40 ◦C for 30 s. The relative expression of genes was calculated by the comparative 2–ΔΔCt method using peptidylprolyl isomerase A (PPIA) RNA levels as an internal control.

Statistical Analysis

Shapiro Wilk ($p > 0.05$) test examined conformity for normal distribution in each group. The data are expressed as mean values ± SD. For normally distributed data, a one-way analysis of variance (ANOVA) was conducted for the probe trial of the MWMT and gene expression followed by Tukey post hoc comparisons (significance determined as $p < 0.05$). Within-subject measurements, such as escape latencies, distance move, and velocity across trials (1., 2., 3., 4., days) in the MWMT, were analyzed using repeated-measures ANOVA. For each day in water maze testing, to test the difference between groups for variables (distance move, latency, and velocity), one-way analysis of variance (ANOVA) was conducted followed by Tukey post hoc comparisons.

RESULTS

The weights of the rats were measured weekly throughout the experimental procedure. There is no significant difference in weight between the groups ($F_{3.28} = 0.924$, $p = 0.444$). The amount of water consumed by the rats daily and the amount of feed consumed weekly were monitored. There was no significant difference between the groups in terms of the amount of water and feed consumed ($F_{3.28} = 2.829$, $p = 0.060$ and $F_{3.28} = 0.712$, $p = 0.554$; respectively).

Morris Water Maze Test

In the MWMT, there was a significant difference in the total distance move between the groups in the 4-day learning phase ($F_{1.28} = 26.958$, $p = 0.000$). There was a significant difference between days 1st compared to the 2nd and 3rd ($p < 0.001$, $p = 0.009$ and $p < 0.001$; respectively), and between days 4th compared to the 2nd and 3rd ($p = 0.001$ and $p < 0.001$, respectively). In the learning phase, the total distance traveled was decreased from the 1st to the 4th day.

There was a significant difference in the time spent

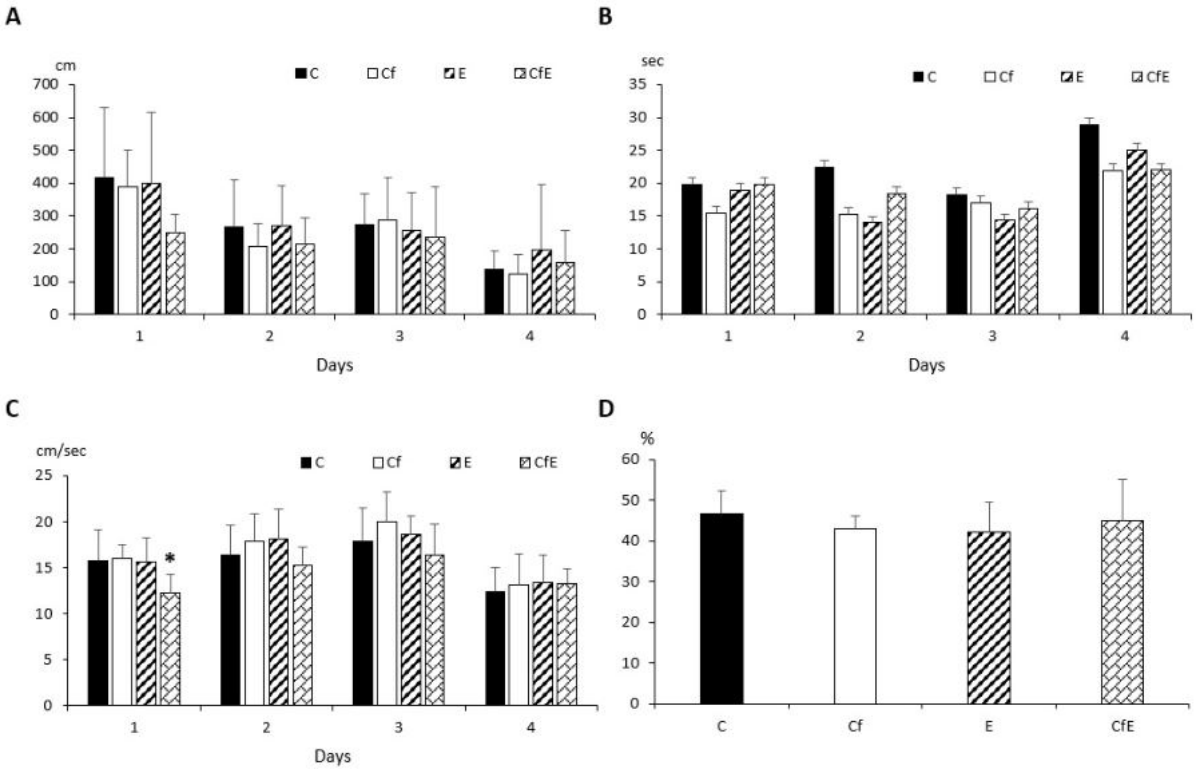

Fig. 1. Mean distance move (cm) (A), escape latency (sec) (B), swimming velocity (cm/sec) (C), and time spent in targeted quadrant (%) (D) in Morris Water Maze Test for C, Cf, E and CfE groups. All values represent the mean ± standard derivation from 7 male rats in each group. * $p < 0.05$, compared with CfE and C, Cf (C).

finding the platform between days. ($F_{1.28} = 5.768$, $p = 0.023$). The time required to find the platform was different from day 1st, 2nd and 3rd day compared to 4th ($p = 0.014$, $p = 0.002$ and $p < 0.001$; respectively). The time required to find the platform was increased on day 4 compared to other days.

There was a significant difference in the swimming speeds between days ($F_{1.28} = 4.360$, $p = 0.046$). Swimming speed has differed significantly between days 1st compared to the 2nd, 3rd and 4th ($p = 0.003$, $p < 0.001$ and $p = 0.009$; respectively), and between days 2nd compared to the 3rd and 4th ($p = 0.005$ and $p < 0.001$, respectively) and between 3rd and 4th days ($p < 0.001$). Swimming speed was decreased on the 4th day.

According to the between-group analysis, there was no significant difference in the total distance move and time to find the platform in the learning phase between groups ($p > 0.05$) (Figs. 1A and 1B). When the swimming speeds of the groups were compared in the learning phase, there was a significant difference only on the 1st day ($F_{3.28} = 3.779$, $p = 0.024$). According to the post-doc analysis, there was a significant difference between the CfE compared to the C and Cf ($p = 0.05$ and $p = 0.038$, respectively). On the 1st day of the learning phase, the swimming speed of the CfE was decreased (Fig. 1C).

When the percentage of time spent in the quadrant with the platform in the test phase was compared, there was no significant difference between the groups ($F_{3.28}= 0.524$, $p = 0.670$) (Fig. 1D).

Open Field Test

In the open field test, there was no significant difference in the total distance move between groups ($F_{3.28} = 2.482$, $p = 0.085$). There was a significant difference in the time spent in the center between groups ($F_{3.28} = 4.402$, $p = 0.014$). CfE spent less time in the center compared to the control group ($p = 0.009$) (Fig. 2A). There was a significant difference in the number of entries to the center between groups ($F_{3,28} = 6.122$, $p = 0.003$). The number of entries to the center was significantly different between the C compared to the E and the CfE. The number of entries was decreased in the E ($p = 0.023$) and CfE ($p = 0.003$) compared to the control group (Fig. 2B).

NR2A, NR2B, and BDNF Gene Expression Levels

NR2A, NR2B, and BDNF gene expression levels in rat hippocampus tissue were determined by the RT-PCR method. In the comparison between groups, there was no significant difference in NR2A ($F_{3.28} = 0.410$, $p = 0.748$), NR2B ($F_{3.28} = 0.017$, $p = 0.997$), and BDNF gene expression levels between the groups ($F_{3.28} = 0.723$, $p = 0.553$) (Figs. 3A, 3B and 3C).

DISCUSSION

Caffeine is an ergogenic supplement that is often preferred because of its stimulant feature in exercise applications, increasing the use of fatty acids and the thought that it will increase performance. In this investigation, the effects of caffeine supplementation on anxiety behavior, hippocampus learning memory function, and NR2A, NR2B, and BDNF levels in rats were examined in a manner that is comparable to the level of moderately taken caffeine in humans. Our results showed that caffeine administered during exer-

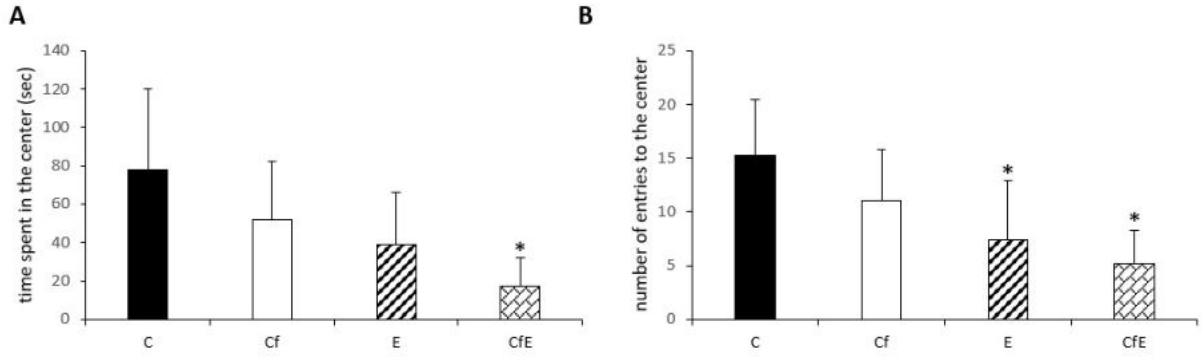

Fig. 2. Comparison of the time spent in the center between groups in the open field test (A). Comparison of the number of entries to the center between groups in the open field test (B). * ($p < 0.05$) compared to the control group. Values were given as mean ± standard deviation.

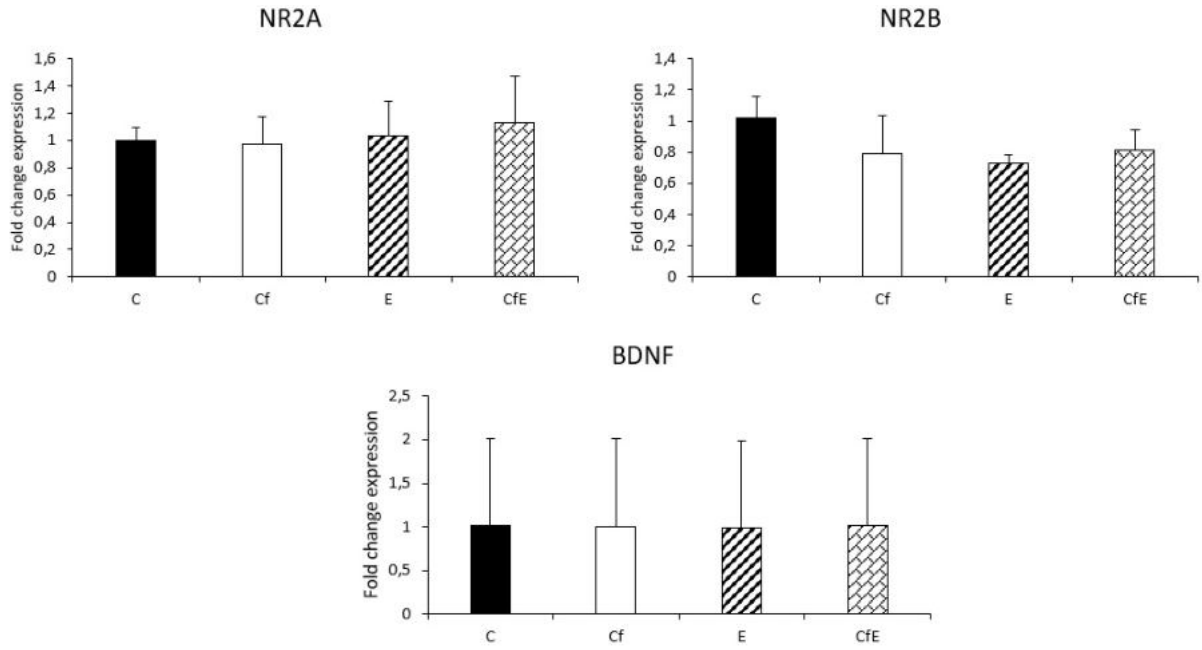

Fig. 3. Graphs showing the levels of fold change gene expression NR2A, NR2B and BDNF. Values were expressed as a percentage of the cage control value (100%) from five male rats in each group. Values were given as mean ± standard deviation; significance level was accepted as $p < 0.05$.

cise caused anxiety behavior but had no significant impact on learning, memory function, or hippocampus gene expressions.

Caffeine can regulate body weight since it is thought to increase energy consumption and affect thermogenesis, fat oxidation, and energy balance [24]. In the caffeine-treated group of obese rats fed a high-carbohydrate, high-fat diet, there was a reduction in body fat (0.5 g/kg caffeine in the final 8 weeks of the 16-week procedure) [25]. In another study, it was concluded that the body weights of rats fed both a high-fat diet and caffeine were not different from the group fed with standard feed and not taken caffeine [26]. In this study, it was determined that given caffeine and applied exercise did not affect the body weight of adolescent male rats. According to another study that supported the findings of the present investigation, caffeine did not affect body weight increase [27]. The effect of caffeine on body weight may be due to differences in administered caffeine dose, nutritional content, gender, body weight, or body fat composition. The response to caffeine may create gender-specific differences depending on circulating steroid hormones [28]. To better understand our findings, more specific studies using gender-specific and individual caffeine dosages are required.

The effects of caffeine on appetite and energy balance are conflicting. Some studies indicate that caffeine has a mild anorectic effect [29]. On the contrary, others demonstrate that caffeine increases appetite in a dose-dependent manner. Low-dose caffeine has been found to significantly increase appetite compared to high-dose caffeine [30]. Furthermore, it has also been demonstrated that caffeine does not affect appetite or food intake [31]. The results of our investigation showed that caffeine did not affect the feed and water intake of the rats.

Differences in caffeine dosage, dietary factors, gender, body weight, or body fat composition may all have an impact on how much caffeine affects body weight. Caffeine, which is widely consumed by humans, affects behavior [32]. Caffeine consumption, especially when excessive, might be problematic for sensitive people. In human tests, caffeine in high (400 mg/day) and moderate (200 mg/day) levels helped people remember more words than caffeine in low doses [33]; beneficial effects were also seen as a caffeine supplement sped up writing [34]. However, a study with university students revealed that caffeine harmed memory since the group that received caffeine remembered fewer words when they took the auditory-verbal learning test [35]. Caffeine administration

to postnatal rats has been proven in studies to have positive benefits on spatial learning [36]. In addition, it was noted that the offspring of rats exposed to caffeine (20 mg/kg twice a day) during pregnancy may suffer from cognitive damage [37]. This study revealed that caffeine did not affect learning and memory performance in MWMT. Similarly, it has been shown that caffeine does not affect learning performance in the new object recognition test [38]. In MWMT, it was found that caffeine treatment (0.3-10 mg/kg) after training increased memory retention while caffeine administration (0.3-10 mg/kg) before training did not affect the performance of the animals [39]. The study by Angelucci *et al*. [39] offers a different perspective on discrepancies in the literature by demonstrating that caffeine improves memory retention but not memory acquisition.

It is well established that physical activity has beneficial benefits on both learning and healthy aging processes. Exercise has been demonstrated to improve the hippocampus and learning and memory processes [40-43]. On the other hand, it has been noted that there is an inverse correlation between memory performance and the intensity of treadmill activity of different intensities [44]. In our study, although it was not statistically significant in MWMT, the time spent in the water to find the platform was the least and the swimming speed was the highest in the exercise group. Our results show that neither caffeine nor exercise had any discernible impact on cognitive function. Similar to our findings, research has demonstrated that caffeine (6 mg/day or 9 mg/day) and exercise have no impact on cognitive function [45], and exercise has no direct impact on cognition [46]. The fact that exercise must be done three days a week and is required may have prevented the potential benefits of exercise from being noticed. Additionally, it's possible that the rats' tolerance was brought on by the administration of caffeine in moderate dosages throughout the experiment.

The open field test is a procedure that assesses an animal's locomotor activity and anxiety by measuring the time they spend in the center and at the margins [47]. The results of a study examining the behavioral effects of regular caffeine consumption in adolescent male rats showed that low (0.1 mg/mL), medium (0.3 mg/mL), and high (1.0 mg/mL) dosages of caffeine had no influence on activity but did have anxiogenic effects, similar to those in our study [48]. In studies examining the effect of caffeine on behaviors like anxiety, the situations such as the dose of caffeine consumed and the sensitivity of the person to caffeine should also be taken into account. In a study, it was demonstrated that caffeine had a dose-dependent effect on anxiety; a high caffeine dose induced anxiety, whereas a low caffeine dose had no such effect [49]. Similarly, other studies have shown that high doses of caffeine cause anxiety symptoms such as inducing panic attacks in Parkinson's patients [50, 51]. Treadmill exercise reduces oxidative stress and anxiety-like behaviors in brain tissue in rats [52, 53]. On the other hand, voluntary wheel exercise was found to be ineffective in reducing anxiety-like behaviors in rats in the study by Jones *et al*. [54]. According to the findings of our study, the time spent in the center of the CfE in the open field test was found to be significantly decreased than the control. In addition, the number of entrances and exits to the center was found to be decreased in the E and CfE compared to the control. This result shows that caffeine alone does not cause anxiety, but there are signs of anxiety in exercise groups. The treadmill used for exercise and the compulsory of exercise may be the cause of anxiety in exercise groups.

NMDA receptors are known to play a role in cognitive functions [55]. It has been determined that chronic use of NR2A antagonists or caffeine supplementation starting at puberty prevents delayed memory deficit and related synaptotoxicity [56]. NMDA receptors have a curative effect on behavioral disorders brought on by caffeine withdrawal [57]. In our study, withdrawal did not occur in rats due to caffeine administration until the experiment was over and there were no alterations in the NMDA receptors. Additionally, there are also conclusions that NMDA-type glutamate receptors do not play a significant role in mediating the locomotor stimulating effects of caffeine or its tolerance to these effects [58].

Several studies are showing that exercise can activate NMDA receptors in the hippocampus [59-61]. The negative effects of maternal stress on depressive-like behaviors in adult rats are mitigated by voluntary wheel exercise during adolescence; stressed rats exhibit an increase in the expression of the NR2A subunit of NMDA receptors in the hippocampus, and its antidepressant-like effects can reduce NR2A expression [62]. In our study, neither caffeine nor exercise changed hippocampal NR2A and NR2B gene expres-

sion levels. These results suggest that wheel exercise may activate NMDA receptors in the hippocampus, which in turn may increase BDNF production and neurogenesis [63].

According to certain research, caffeine improves memory functions by increasing BDNF levels in the hippocampus [64, 65]. On the other hand, exposure to caffeine (20 mg/day twice daily) during pregnancy has been observed to result in decreased BDNF levels in the offspring [37]. Although voluntary chronic exercise is known to increase BDNF levels in rats, the effects of compulsive wheel exercise are not clear. The intensity of the run is another component that contributes to this. In a study where acute wheel exercise was used at different running intensities, it was found that low-intensity (15 m/min) wheel exercise which generates minimum stress, can increase BDNF and hippocampus functioning in comparison to more severe versions [66]. It has been observed that compulsory exercise (starting with a 3-minute warm-up at 8 m/min and gradually increasing to 12 m/min for 30 minutes on training days after 10 m/min in 10 minutes in the first sessions) which were subjected to pregnant rats increased hippocampal BDNF levels [67]. Additionally, it was found that moderate-intensity treadmill activity (2 weeks, 20 minutes per day) had no effect on the BDNF levels in the regions of the brain under investigation [68]. Our findings on hippocampus NR2A, NR2B, and BDNF appear to be in line with the conclusion that MWMT does not affect memory and learning. A moderate dose of caffeine and compulsory exercise does not affect learning and memory performance and related gene expression level either alone or together.

CONCLUSION

According to our findings, chronic exposure to caffeine and compulsory exercise in the early life do not have a significant effect on learning and memory functions. Caffeine and compulsive exercise appear to have a major effect on anxiety behavior. In our study result of, caffeine supplementation and exercise have no effect on learning and memory behavior in MWMT and no effect on learning-related genes in the hippocampus are consistent findings with each other. The chronic administration of moderate doses of caffeine and the obligatory 3 days per week of exercise are thought to be the main factors affecting the results of the study. As a result, it is noteworthy that the interactions between caffeine and exercise are unpredictable and the complexity of their effects on memory functions. In order to eliminate the acute effect of exercise after chronic exercise, tests should be performed at least 24 hours after the last application. In conclusion, to better understand how caffeine and exercise affect memory and learning, more studies are required that simultaneously address a variety of variables, including caffeine dose, chosen age group, gender, exercise to be performed (compulsory/voluntary), exercise duration, tests to be subjected, and other parameters.

Authors' Contribution

Study Conception: SMK, FB; Study Design: SMK, FB, LS; Supervision: SMK; Funding: SMK; Materials: SMK, FB, OSC, LS; Data Collection and/or Processing: SMK, FB, LS, OSC; Statistical Analysis and/or Data Interpretation: SMK, FB, LS; Literature Review: SMK, FB; Manuscript Preparation: SMK, FB, OSC, LS and Critical Review: SMK, LS.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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